#2

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Request for grant of a patent

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1. Your reference — 4-32652P1/NEI 8027

2. Patent application number *(The Patent Office will fill in this part)* — 0228429.7 — -5 DEC 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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LICHTSTRASSE 35
4056 BASEL
SWITZERLAND

Patent ADP number *(if you know it)* 7125487005

If the applicant is a corporate body, give the country/state of its incorporation — SWITZERLAND

4. Title of invention — Organic compounds

5. Name of your agent *(If you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)* 1800001

6. If you are declaring priority from one ore more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing (day/month/year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

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(see note (d))

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	One
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application

Signature: B. A. Yorke & Co.

Date: 05 December 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Mrs. J. Crook
020 8560 5847

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DUPLICATE

Organic Compounds

The present invention relates to organic compounds, e.g. for use in high-throughput screening.

A broad range of biological and high-throughput screening assay applications are based on selective detection of a target molecule, e.g. protein, by attachment of an appropriate label. This is usually achieved by incorporation of the label via an appropriate reactive group which allows selective reaction with certain groups on the target protein (e.g. reaction of a succinimidylester-activated dye with primary amines in the sidechains of a protein). However, such conventional labeling approaches may face several major drawbacks: firstly, depending on the amino acid sequence of the target protein, several groups may have the potential to react with the labeling reagent. This leads to the generation of a mixed population with random combinations of stoichiometries and incorporation sites. However, a majority of applications requires a homogeneous population of the molecules under investigation with defined stoichiometries and positions of the label. Secondly, the limited efficiency of most chemical reactions results in a certain percentage of remaining unreacted substance, which usually interferes with the measurement and should be quantitatively removed. As the biochemical properties of the molecule should preferably not be dramatically changed by the introduction of such modification, the separation of the two species by common biochemical methods is often not straightforward. Thirdly, each molecule requires its own, optimised labeling strategy. In today's HTS environment, where a vast number of target molecules need to be prepared in parallel, ideally based on a common approach, this forms a significant hurdle.

We have now established a generic labeling technology for generating highly pure, e.g. 100%, 1:1 labeled target protein or peptide, e.g. recombinant protein, applicable to virtually any desired label and target protein or peptide sequences.

The approach according to the present invention is based on the specific ligation of a target protein or peptide to a residue allowing the positioning of the label and separation from unreacted target protein or peptide.

In one aspect the present invention provides a compound of formula

A-Y-D-Y′-X-Y′′-E I

wherein

A is an amino acid residue or a chemical reactive group,

D is a label bearing residue,

X is present and is a linker residue allowing cleavage of compounds of formula I to liberate A-Y-D-Y′ fragment with Y and/or Y′being present or not, respectively, or is not present,

Y, Y′ and Y′′ is/are present and independently of each other are a spacer residue or Y and/or Y′and/or Y′′ is/are not present, and

E is an affinity tag residue.

"Residue" is understood to be a chemical item, e.g. an amino acid residue in case of A, which is bound to one or more other chemical items, e.g. D in case of A or Y and Y or Y′ in case of D.

A is an amino acid residue or a reactive chemical group. In a preferred aspect the amino acid residue is a cysteine residue and the reactive chemical group is one selcted from the group consisting of thioester, aldehyde, maleimide, vinyl and haloalkylcarbonyl.

D is a label bearing residue, preferably an (oligo)peptide bearing a label, e.g. a dye label, preferably a fluorescent dye label.

X is a linker residue originating from groups known to be useful as linkers in combinatorial, peptide or oligonucleotide chemistry which comprise the ability to covalently bind to D, Y′and/or E. Such linkers include e.g. photocleavable linkers (= a linker cleavable by light, also called light labile linker), chemically cleavable linkers like e.g. base labile, preferably acid labile linkers, enzyme-cleavable linkers, redox-labile linkers and masked linkers, e.g. benzyl, benzhydryl, benzhydrylidene, trityl, xanthenyl, benzoin, silicon or allyl based linkers. Conventionally a photocleavable linker may carry e.g. a nitroaryl group, such as e.g. 4-alkyl-5-nitro-benzoyl or α-methyl-6-nitroveratryl groups. An acid cleavable linker may comprise a Rink Amide (RAM) or very acid labile 2-chlorotitryl linker. Such linkers are known and may be prepared as appropriate.

X may also not be present. When X is not present the affinity tag residue is in a preferred aspect a tag which binds reversible to a support material, e.g. a solid phase.

E is an affinity tag which may bind either reversibly or more or less irreversibly to a support material, e.g. a solid phase, preferably a coated (solid) support material. The affinity tag comprises preferably a substance selected from the group consisting of amino acid sequence, preferably 4 to 10 amino acids, amino acid and biotin. The support material preferably comprises a substance selected from the group consisting of amino acid chelating agent, e.g. nickel-ion, anti-amino acid sequence (= complementary to the amino acid sequence of the affinity tag), avidin and streptavidin. In a preferred aspect the following pairs for substances are comprised in the affinity tag and in the support material: FLAG (=DYKDDDDKGK) – anti-FLAG antibody,
His_6 – Ni^{2+}-NTA, biotin – avidin or biotin-streptavidin.

Y and/or Y` and/or Y`` is/are present and independently of each other are a spacer residue. A spacer residue e.g. should facilitate the control of cleavage of the linker, thereby separating e.g. the A-D fragment of a compound of formula I or separating the linker more efficiently from the rest of the compound. Preferably the spacer residue originates from substances known to be useful as spacer residues in combinatorial, peptide and oligonucleotide chemistry, with the ability to bind, e.g. covalently, to A, D, X and/or E, respectively. A spacer residue can also be comprised in the label bearing residue D and therefore may also have the ability to bind to any binding partner of the label bearing residue D. In a preferred aspect the spacer residue Y`` is present, e.g. a spacer residue between the cleavable linker and the affinity tag is present. A spacer comprises a natural or synthetic amino acid or a long chain substance like e.g. aminoalkyl, aminoalkoxy, aminoalkylcarboxylic acid or aminooxa-alkylcarboxylic acid containing group, preferably the spacer residue is diaminoalkyl, glycine, β-alanine, 4-aminobutyric acid or 8-amino-3,6,-dioxaoctanoic acid. The spacer may be present in single or multiple copies. The spacer may comprise solubility enhancing residues and/or hydrophilic residues.
Y and/or Y` and/or Y`` may also not be present.

The target protein or target peptide is the molecule which is to be labeled. It may be any (bio)chemical substance comprising amino acids, such as e.g. a receptor molecule, an enzyme, an antigen, an antibody or any other substance of interest. The target protein or target peptide can be isolated from a biological source or can be chemically or recombinantly produced. For binding (ligating) the protein or peptide to a compound of formula I an amino acid residue, e.g. a cysteine, may be introduced into the target protein or target peptide by

methods as conventional, e.g. by chemical or recombinant methods or, such cysteine may be already present.

The binding (ligation) of a target protein or target peptide to a compound of formula I can be carried out according to methods as conventional. E.g. ligation can be achieved by reaction
a) of a C-terminal thioester of a target protein or target peptide with an N-terminal cysteine as the chemical reactive group in A (see e.g. figure 1),
b) of a thioester of a cysteine containing residue in A with an N-terminal cysteine containing target protein or target peptide, or
c) of a cysteine containing target protein or target peptide (C-terminal or within the protein or peptide) via maleimide to a cysteine containing residue in A of a compound of formula I.

In providing a compound of formula I it is possible to have any target protein or target petide 1:1 labeled. With an affinity tag coupled e.g. via a cleavable linker to the labeled target protein or target peptide, a labeled compound may be separated from unlabeled compound in that the labeled compound is bound to an affinity support whereas the unlabeled compound is not bound. After cleavage of e.g. the linker X only labeled target protein or target peptide are obtained as e.g. products obtained from coupling (ligation) of target protein or target peptide to a compound of formula I.

In another aspect the present invention provides a method for identifying an agent that modulates the activity or characteristic of a target protein comprising:
a) providing a compound of formula I,
b) providing a target protein or a target peptide,
c) coupling the compound of a) to a target protein or target peptide of b) so that a coupled product is obtained,
d) contacting the coupled product with a candidate compound, which is expected to modulate the activity or characteristics of the target protein or target peptide,
e) measuring a signal in the absence and in the presence of a candidate compound and determining whether there is a difference in the measured signals, and
f) choosing an agent determined in a) to e), e.g. for use as a pharmaceutical.

A candidate compound includes compound(s)(libraries) from which its influence on the target protein or target peptide can be determined. Compound (libraries) include for example

oligopeptides, polypeptides, proteins, antibodies, mimetics, small molecules, e.g. low molecular weight compounds (LMW`s).

An agent is a compound which modulates, e.g. inhibits, the activity or influences the characteristics of a target protein or target peptide.
An agent is one of the chosen candidate compound. An agent includes one or more agents.

In another aspect the present invention provides the use of a compound of formula I in a high-throughput screening assay.

In a further aspect the present invention provides a kit comprising a compound of formula I and instructions for using the kit.

Said kit may further comprise a substantial component including an appropriate environment of a sample to be tested and, e.g. appropriate means to determine the effect of a candidate compound in a sample to be tested.

In still another aspect the present invention provides a method for identifying an agent that modulates the activity or characteristics of a target protein or peptide comprising the use of a compound of formula I, e.g. in an assay of the present invention.

Description of the FIGURES:

Figure 1: Schematic representation of a compound of formula I comprising a chemical reactive group (A), a label bearing residue (D), a linker residue (X) and an affinity tag (E) for generic C-terminal, 1:1 labeling of a target protein.

Figure 2: Transesterification reaction between the C-terminal thioester of a target protein and the N-terminal cysteine containing compound of formula I (a) and intramolecular S-N Acyl shift resulting in the formation of a stable amide bond (b).

Figure 3: A specifically applied design of a compound of formula I comprising an N-terminal cysteine as the chemical reactive group (A), a lysine branchpoint for dye attachment (D), a photocleavable linker (X) and a C-terminal affinity tag (E), e.g. Biotin, His_6, FLAG. The compound can further contain a hydrophilic spacer of variable length (e.g. 8-amino-3,6-oxaoctanoic acid).

Figure 4: Formula of the compound of example 4.

Figure 5: LC/ESI-MS of the purified compound of example 4.

Figure 6: Formula of the compound of example 16

In the following examples all temperatures are in degree centigrade and are uncorrected. RT means room temperatures.

The following ABBREVIATIONS are used:

ADO spacer	8-amino-3,6-dioxaoctanoic acid (Neosystem)
Alexa488-maleimide	reactive maleimide derivative of fluorescent dye Alexa488™ (Molecular Probes)
Cys	cystein
Cy5-maleimide	reactive maleimide derivative of fluorescent dye Cy5 (Amersham Pharmacia Biotech)
DTT	dithiothreitol
DIC	N,N´- diisopropylcarbodiimid
DIPEA	diisopropylethylamin
FF	fast flow
FLAG	DYKDDDDKGK amino acid sequence in one letter code
His_6	hexa-histidine
HOBt	1-hydroxybenzotriazol
HTS	high throughput screening
HuR	Hu antigen R (antigen present in patients with Hu-syndrome)
Hu-syndrome	paraneoplastic encephalomyelitis sensory neuropathy
HuR12	variant of HuR antigen
LC/ESI MS	liquid chromatography/electrospray injection mass spectrometry
2-MESNA	2-mercaptoethanesulfonic acid
NTA	nitrilotriacetic acid
PyBop	(benzotriazol-1-yloxy)-tripyrrolidinophosphoniumhexafluoro-phosphate
RAM	Rink Amide (type of resin esp. useful in solid phase techniques)
RP-HPLC	reversed phase HPLC
TBTU	O-(benzotriazol-1-yl)-N,N,N`,N`-tetramethyluroniumtetra-fluoroborate
TMR-maleimide	reactive maleimide derivative of fluorescent dye Tetramethylrhodamine (Molecular Probes)

EXAMPLES

1. Compound of formula I (peptide reagent):

In the following examples a compound of formula I, e.g. a peptide reagent, is defined as a compound (also called construct) comprising the following structures: A which comprises a chemical reactive group, D as a label bearing residue, an affinity tag E, optionally a linker residue X and optionally spacer residues Y and/or Y' and/or Y'' between A and/or D and/or X. Suitable reagents (as given in e.g. figure 3, also without a variable spacer residue) with peptidic backbone as the label bearing residue D are prepared by stepwise synthesis on solid support (e.g. Tentagel-S-RAM-resin) using general peptide coupling chemistry with DIC/HOBt or TSTU/DIPEA activation and protected or modified amino acids (see e.g. Chan W.and White P.D., Basic procedures: Fmoc solid phase synthesis, A practical approach, 41-76, 200, New York, Oxford University Press). Protected aminoalkoxycarboxylic acids or e.g. aminoalkyl-nitroaryl-alkoxycarboxylic acids are introduced as additional spacer residues Y, Y'and/or Y'' or as cleavable linker residue X. Selective deprotection allows introduction of fluorescent dyes as carboxylic acid with PyBop/DIPEA activation or as activated esters into side chains. A crude compound of formula I (peptide reagent) is obtained in cleaving off from the solid support where the compound is synthesised. The crude compound or reagent can be further purified, e.g. chromatographically.

2. GENERAL SCHEME for labeling of a target protein or target peptide and purification of the labeled protein or peptide:

a) The target protein or target is prepared e.g. in the IMPACT™-CN-or IMPACT™ – TWIN System [New England Biolabs], which generates a native but thioester-activated C-terminus of the protein or peptide.

b) Transesterification of the generated C-terminal thioester with the N-terminal cystein of an appropriately selected compound of formula I (peptide reagentI) occurs by nucleophilic attack of the sulfur of the Cys–SH group to the carbon in the thioester group (see figure 2a). The unstable peptide reagent-protein thioester or peptide reagent-peptide thioester intermediate is drawn out of equilibrium by an intramolecular shift to a stable native amide bond (figure 2b), which (i) shifts the equilibrium towards the fusion product and allows a nearly quantitative reaction and (ii) directs the ligation towards exclusive reaction with an N-terminal cystein.

c) Unreacted peptide reagent is removed, e.g. by size exclusion chromatography or by dialysis.

d) Remaining unlabeled target protein or target peptide is removed by affinity chromatography using the affinity tag of the compound of formula I (peptide reagent). This can be based on a reversible affinity interaction such as His_6 with Ni^{2+} or an "irreversible" interaction such as biotin with streptavidin or avidin, or a direct covalent bond to a solid support.

e) Labeled protein or labeled peptide is recovered from the affinity column and the affinity tag is removed by specific cleavage of the linker, either by photophysical or by chemical means.

f) optionally hydrophilic spacing residues of variable length are present between e.g. the cleavable linker and the affinity tag for e.g. optimization of positions and solubility properties.

3. Labeling of the protein HuR12 as a target protein:

For site-specific labeling of the C-terminal thioester of HuR12 a chemically highly stable thioester is generated with 2-MESNA. Accordingly, LC/ESI MS reveals more than 98% of the protein being still present as C-terminal thioester after several purification steps (affinity chromatography, preparative RP-HPLC, lyophilization). In contrast, thioesters prepared with DTT are already hydrolysed to more than 60% after analogous treatment.

The procedure is typically performed as follows:

a) Coupling of HuR12-2-MESNA to e.g. a substance of example 4 (see figure 4) at a peptide reagent-to-target protein ratio between 5:1 and 25:1 and pH 8.0 in presence or absence of 50mM 2-MESNA for 15 hours at 4°C, protected from light.

b) Purification

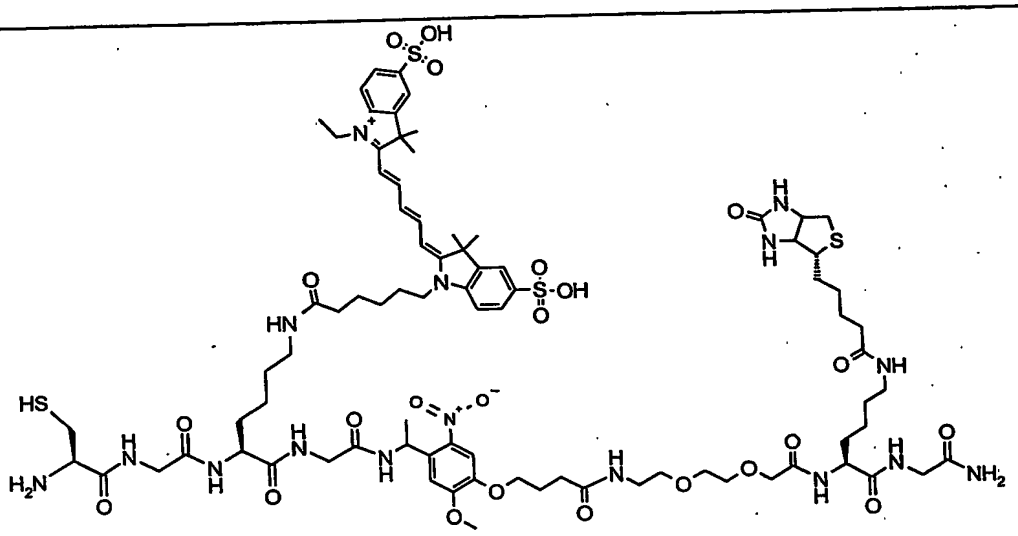
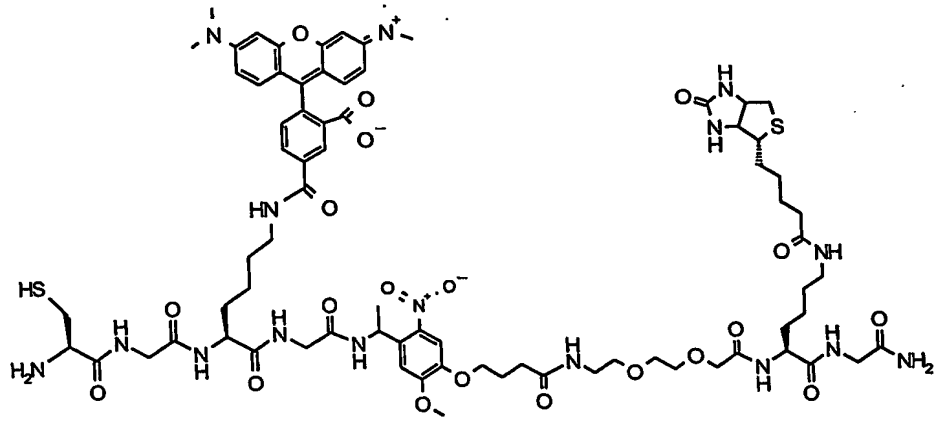
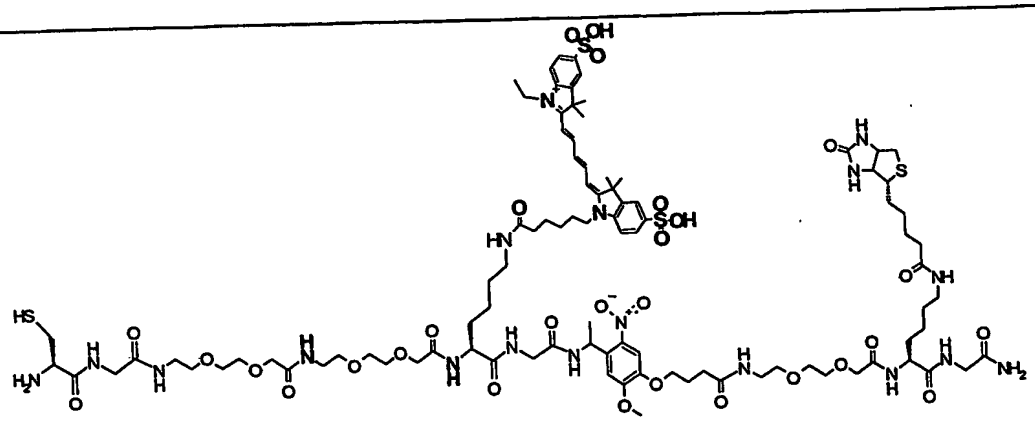
Unbound peptide reagent is removed by gel filtration (e.g. BioRad DG10 columns) and unlabeled target protein is removed via immobilisation of the affinity tagged protein to Streptavidin Sepharose FF [AP Biotech].

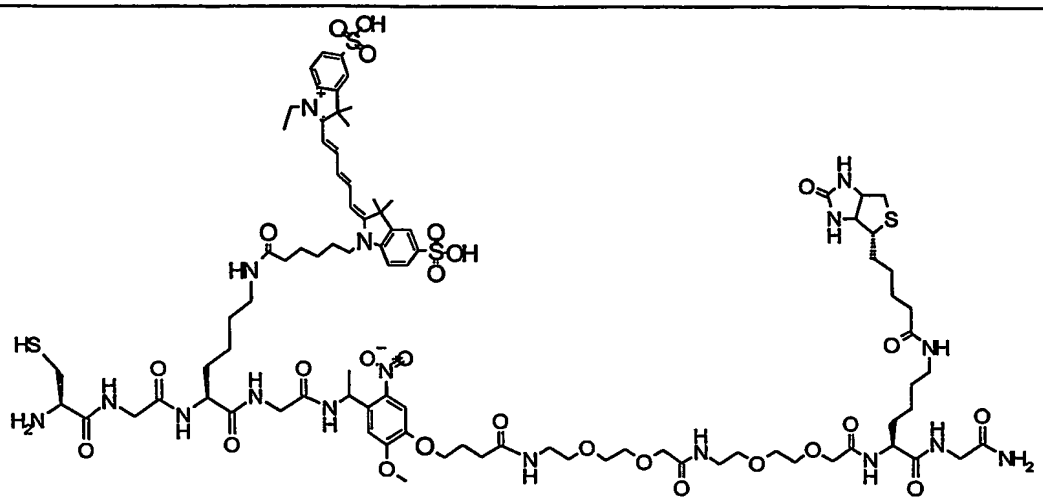
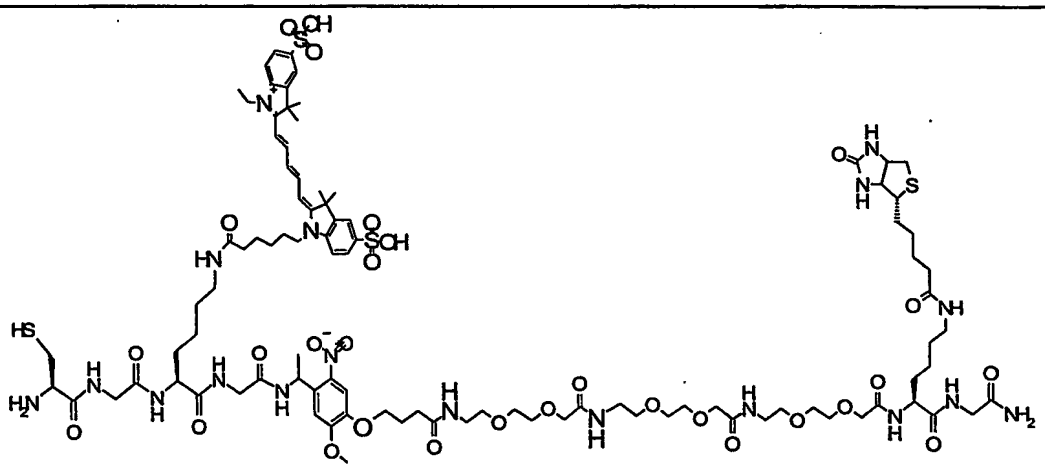
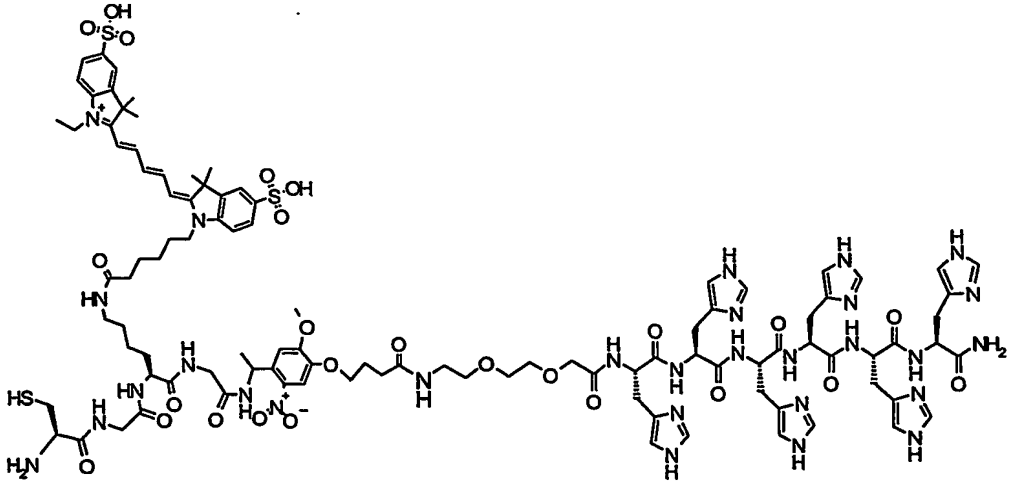
c) Cleavage of the linker

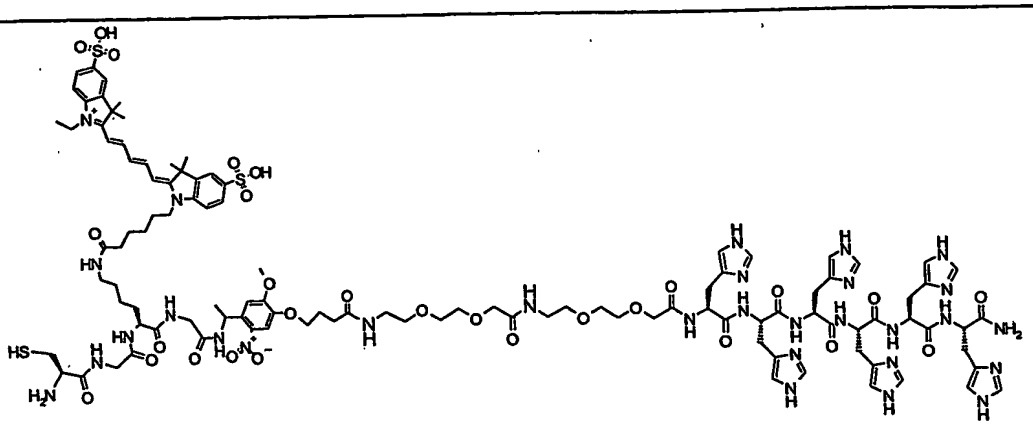
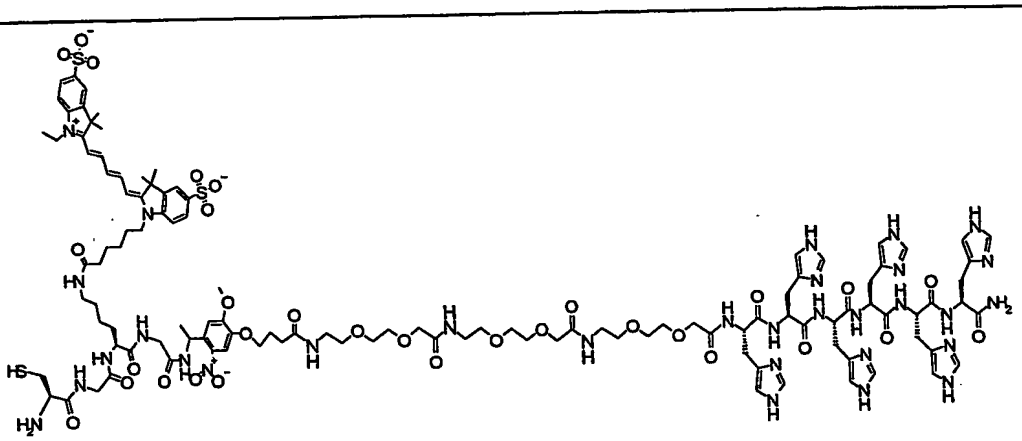
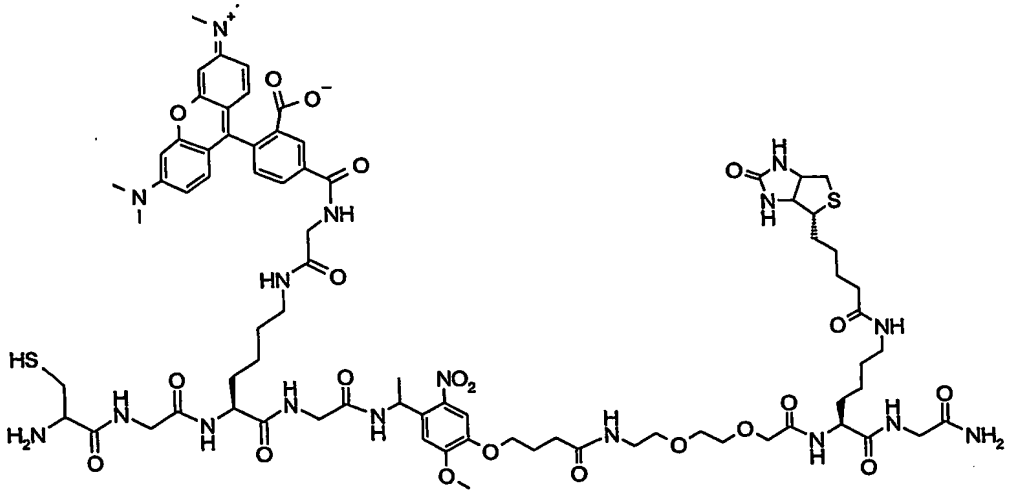
Labeled target protein is recovered by in-column photocleavage of the linker residue for 60 min at 365 nm, 3 mW/cm² [UV STRATALINKER 1800] in a glass vial and under stirring.

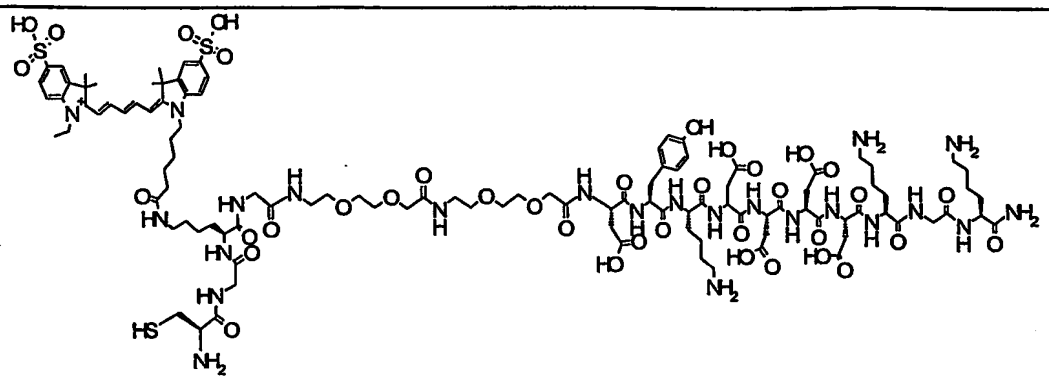
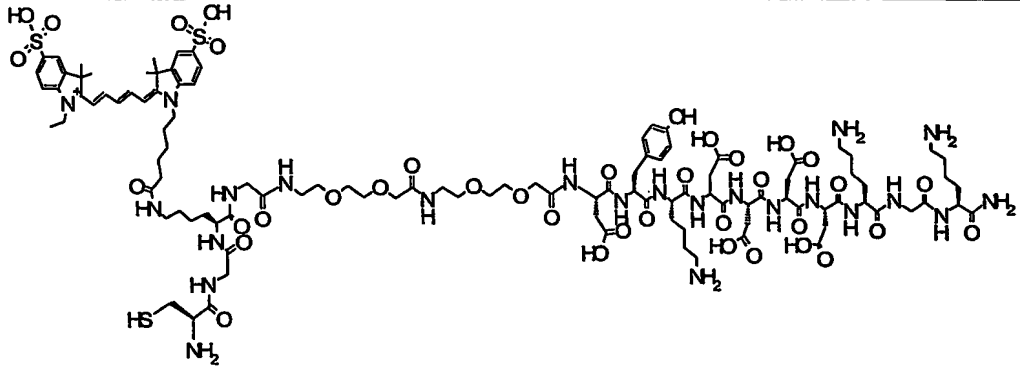
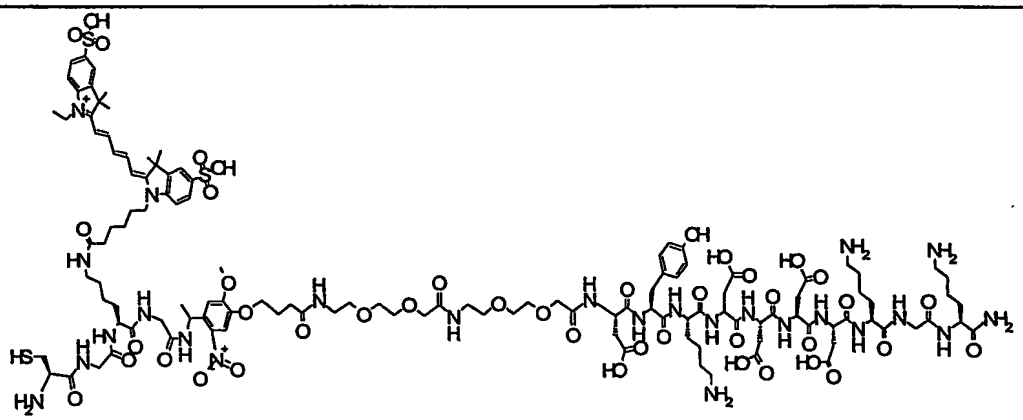
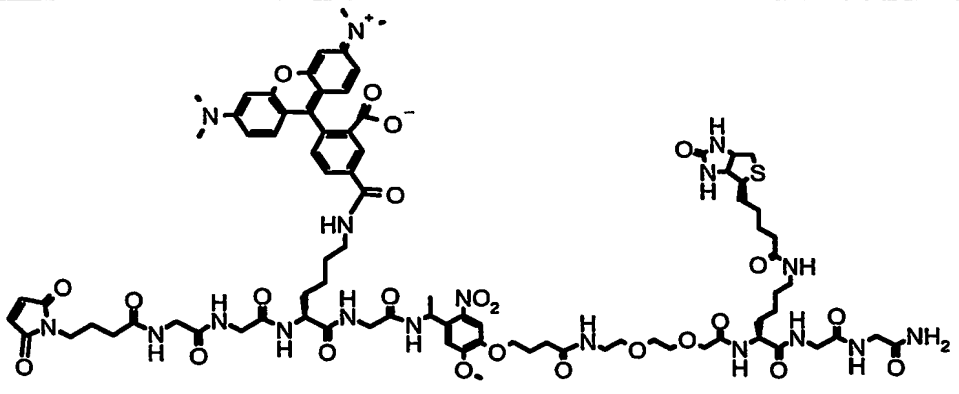
A 1:1 labeling stoichiometry and >99% purity for the labeled protein is confirmed by LC/ESI-MS (figure 5) and RP-HPLC analysis, whereas CD-spectroscopy reveals that the secondary structure of the protein is not affected by the affinity tag as compared to the CD-spectrum of the unlabeled protein (see figure 6).

According to the method as described in example 1 or 2, but using the appropriate starting materials, the following compounds are obtained:

Example	Structure
4	
5	
6	

7	
8	
9	

10	
11	
12	

13	
14	
15	
16	

Example 16:

Compound of example 16 is obtained and coupled to a target protein as follows:

a) the target protein is provided in native form as a thioester at the C-terminus.

b) A peptide reagent as described in example 1 but with a maleimide group as the reactive group instead of the cysteine residue is coupled to a) so that a stable thioether is formed (see figure 7).

Patent claims

1. A compound of formula

A-Y-D-Y′-X-Y′′-E I

wherein
A comprises a chemical reactive group,
D is a label bearing residue,
X is present and is a linker residue allowing cleavage of compounds of formula I to liberate A-Y-D-Y′ fragment with Y and/or Y′ being present or not, respectively, or X is not present,
Y and/or Y′ and/or Y′′ is present and independently of each other is a spacer residue or Y and/or Y′and/or Y′′ is not present, and
E is an affinity tag residue.

2. A compound of formula I according to claim 1 wherein the chemical reactive group is one selected from the group consisting of amino acid residue, e.g. a cystein residue, thioester, aldehyde, maleimide, maleimido-carboxylic acid, vinyl and haloalkylcarbonyl.

3. A compound according to claim 1 or 2 wherein the label bearing residue D is an amino acid or a peptide bearing a label, e.g. a fluorescent dye label.

4. A compound according to any one of claim 1 to 3 wherein the linker X is present and is a photocleavable linker, e.g. a nitroaryl containing linker, or a chemically cleavable linker, preferably an acid labile linker.

5. A compound according to any one of claims 1 to 4 wherein the spacer residue comprises an amino acid, aminoalkyl, aminoalkoxy, aminoalkylcarboxylic acid or aminooxaalkyl-carboxylic acid.

6. A compound according to any one of claims 1 to 5 wherein the affinity tag E binds reversible or irreversible to a solid support.

7. A compound according to any one of claims 1 to 6 wherein the affinity tag E comprises a substance selected from the group consisting of amino acid, (oligo)peptide, biotin and combinations thereof and the solid phase is a substance selected from the group consisting

of amino acid chelating agent, anti-(oligo)peptide of the affinity tag, avidin, streptavidin and combinations thereof.

8. A method for identifying an agent that modulates the activity or characteristic of a target protein or target peptide comprising:

a) providing a compound according to any one of claims 1 to 7,

b) providing a target protein or a target peptide,

c) coupling the compound of a) to a target protein or target peptide of b) so that a coupled product is obtained,

d) contacting the coupled product with a candidate compound, which is expected to modulate the activity or characteristics of the target protein or target peptide,

e) measuring a signal in the absence and in the presence of a candidate compound and determining whether there is a difference in the measured signals, and

f) choosing an agent determined in a) to e), e.g. for use as a pharmaceutical.

9. An assay for high throughput screening comprising a compound of claim 1.

10. A kit comprising a compound according to any one of claim 1 to 7 and instructions for using the kit.

IL/4-Dec-2002

Abstract

A compound of formula

A-Y-D-Y′-X-Y′′-E I

wheren A, D, X, Y, Y′, Y′′ and E have the various meanings as described.

IL/4-Dec-2002

Figure 1

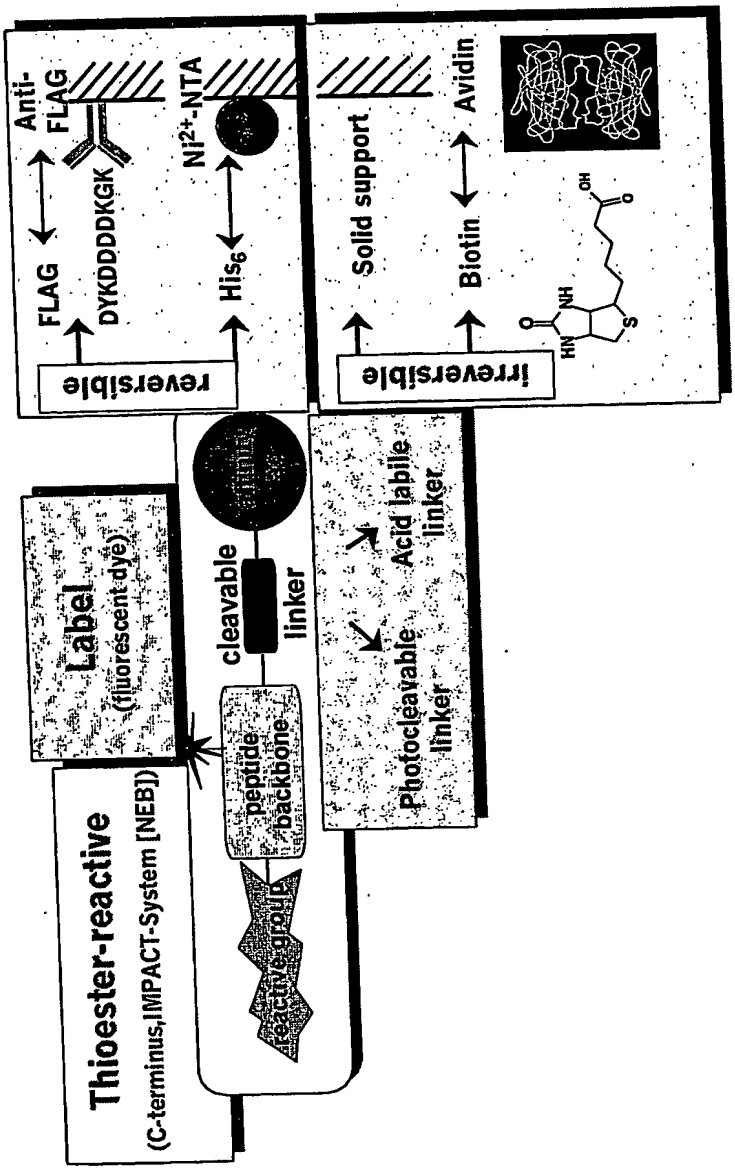

Anti-FLAG
FLAG
DYKDDDDKGK
Ni^{2+}-NTA
His_6
reversible
Solid support
Avidin
Biotin
irreversible
Label
(fluorescent dye)
cleavable linker
Acid labile linker
Photocleavable linker
peptide backbone
reactive group
Thioester-reactive
(C-terminus, IMPACT-System [NEB])

Figure 2

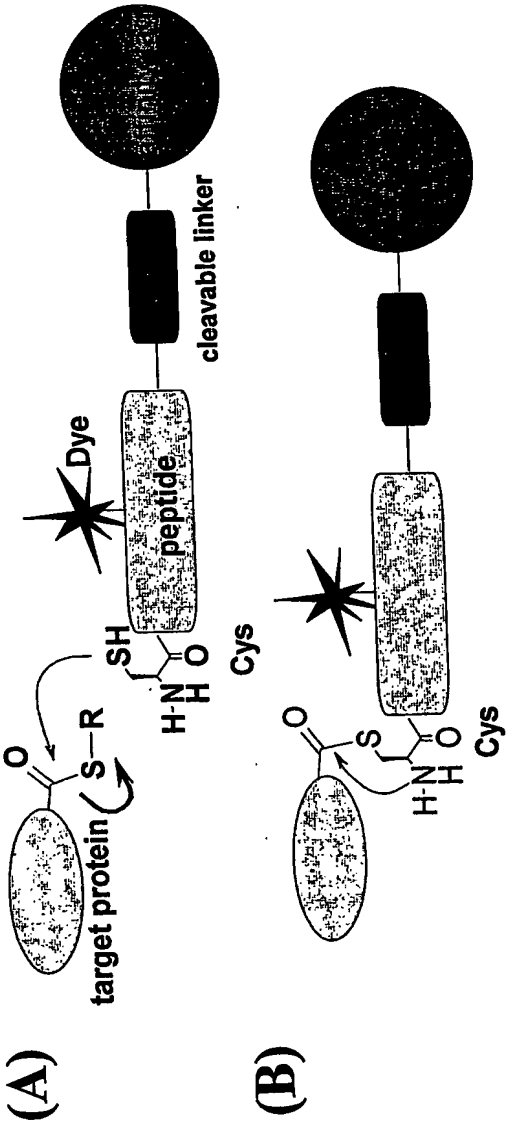

cleavable linker
Dye
peptide
SH
Cys
H-N
H
O
O
S
R
target protein
(A)
(B)
O
S
H-N
H
O
Cys

Figure 3

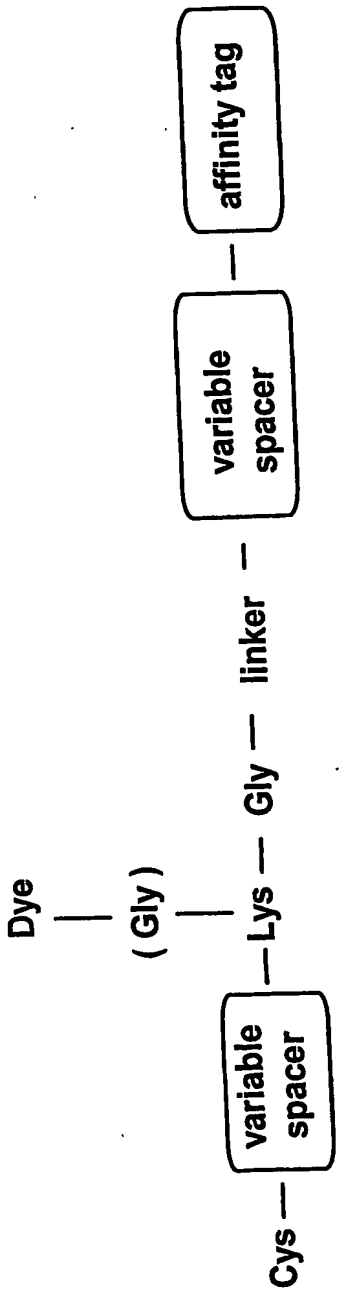

Dye
(Gly)
Lys
Gly
linker
variable spacer
affinity tag
Cys
variable spacer

Figure 4

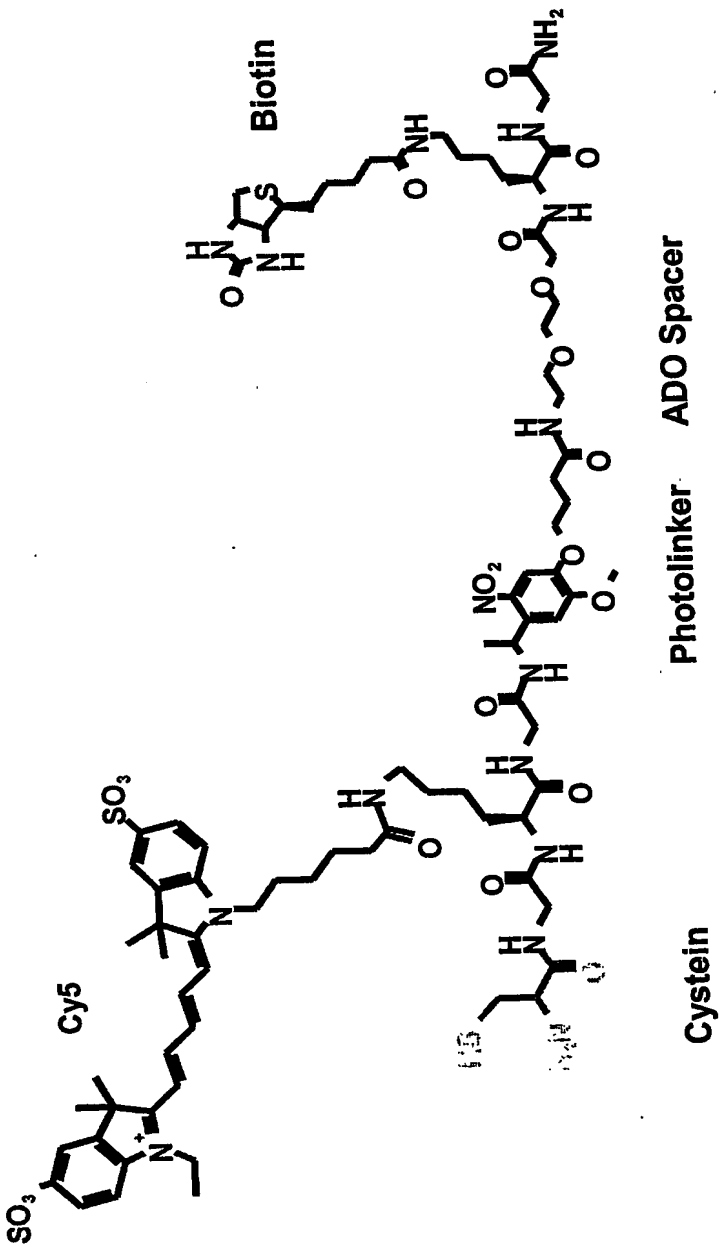


Figure 5

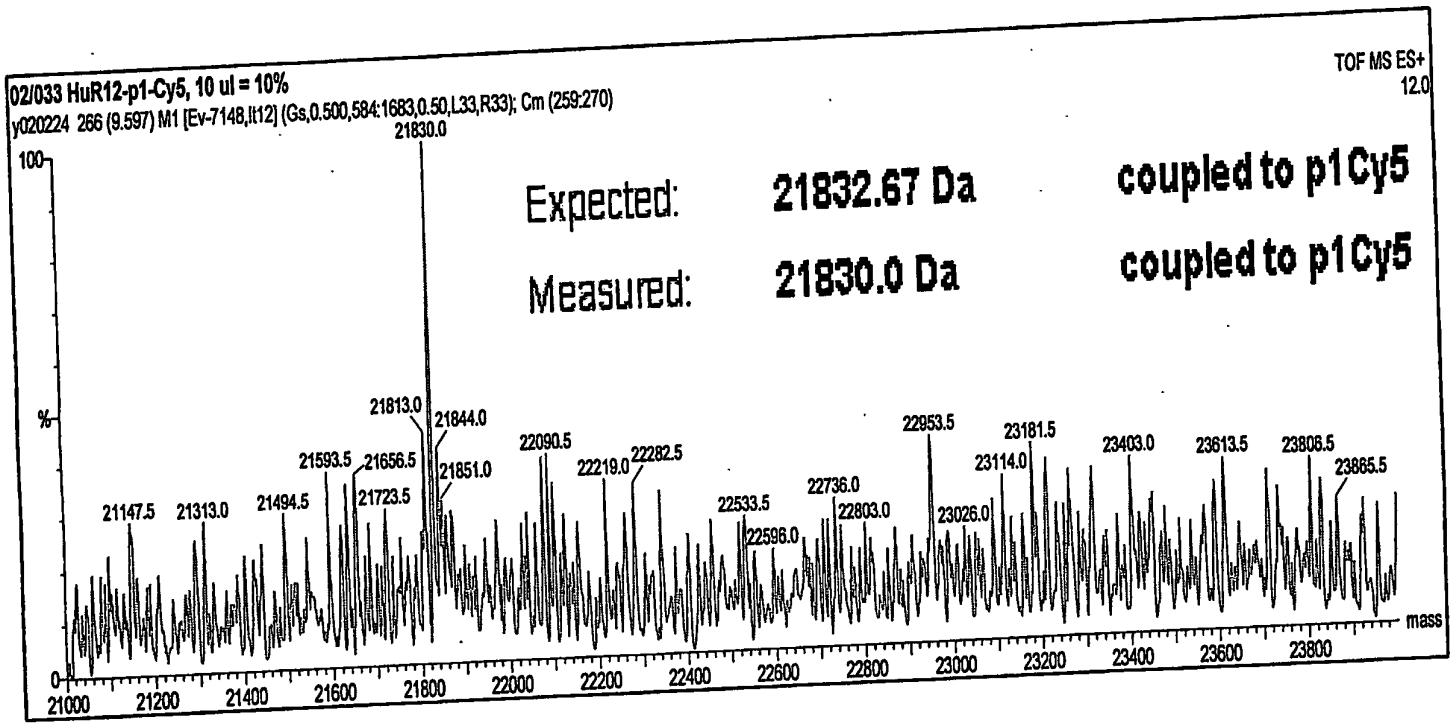

02/033 HuR12-p1-Cy5, 10 ul = 10%
y020224 266 (9.597) M1 [Ev-7148,It12] (Gs,0.500,584:1683,0.50,L33,R33); Cm (259:270)
TOF MS ES+
12.0
Expected: 21832.67 Da coupled to p1Cy5
Measured: 21830.0 Da coupled to p1Cy5
100
%
0
mass
21000
21200
21400
21600
21800
22000
22200
22400
22600
22800
23000
23200
23400
23600
23800
21830.0
21813.0
21844.0
21851.0
21593.5
21656.5
21723.5
21494.5
21313.0
21147.5
22090.5
22219.0
22282.5
22533.5
22596.0
22736.0
22803.0
22953.5
23026.0
23114.0
23181.5
23403.0
23613.5
23808.5
23865.5

Figure 6

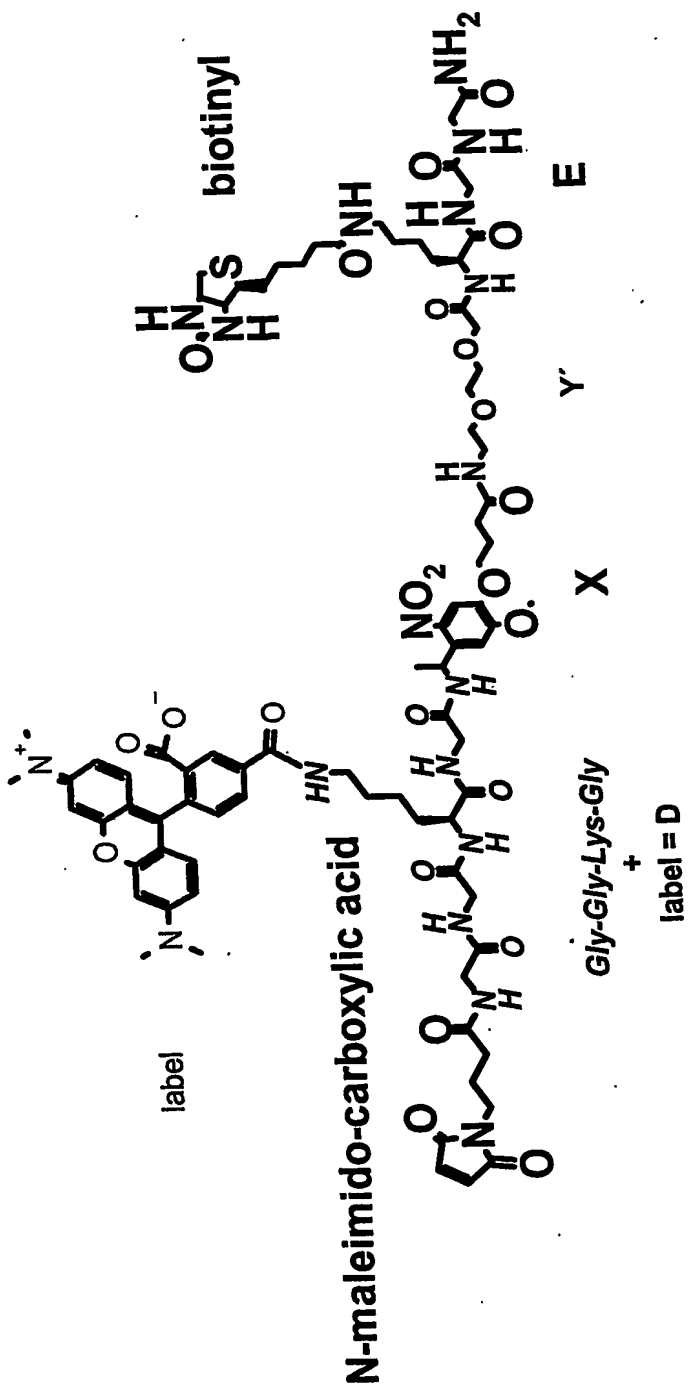

biotinyl
E
Y'
X
label
N-maleimido-carboxylic acid
Gly-Gly-Lys-Gly
+
label = D

Figure 7

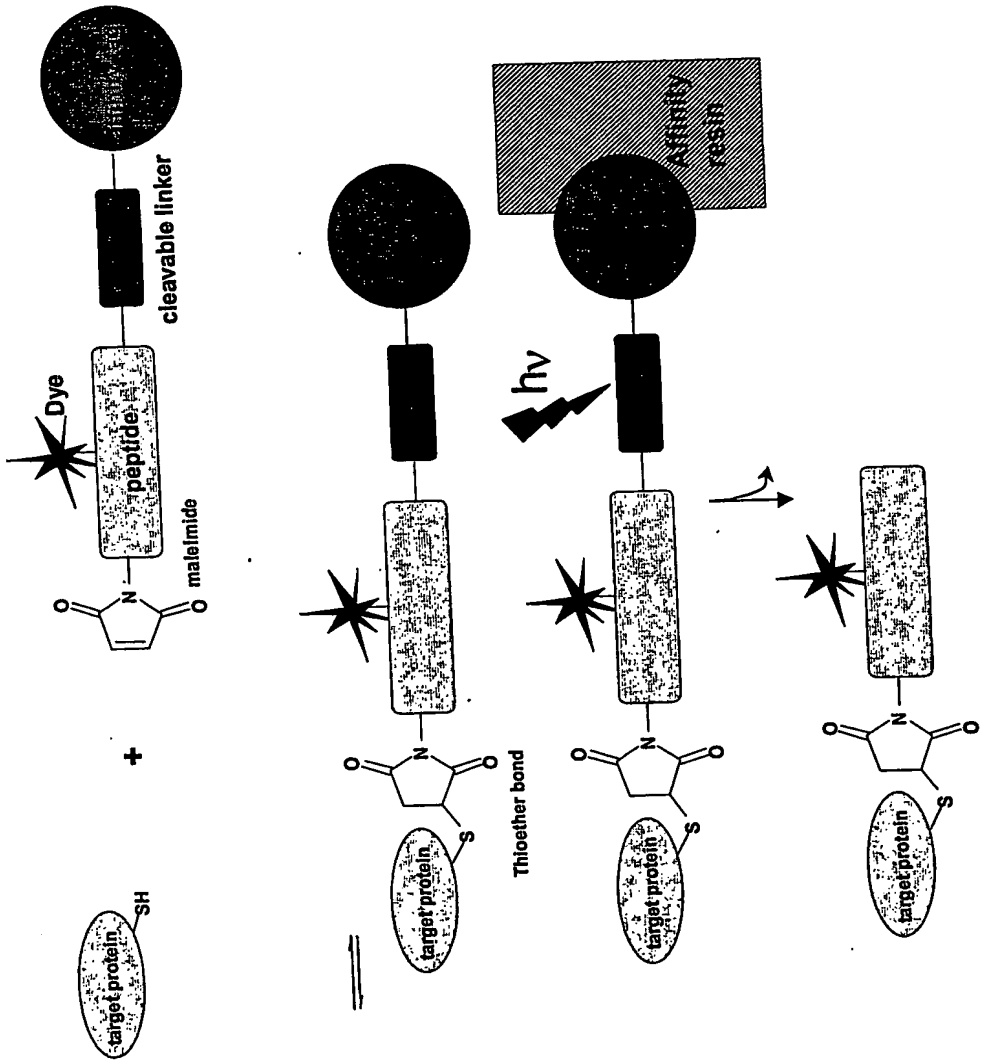
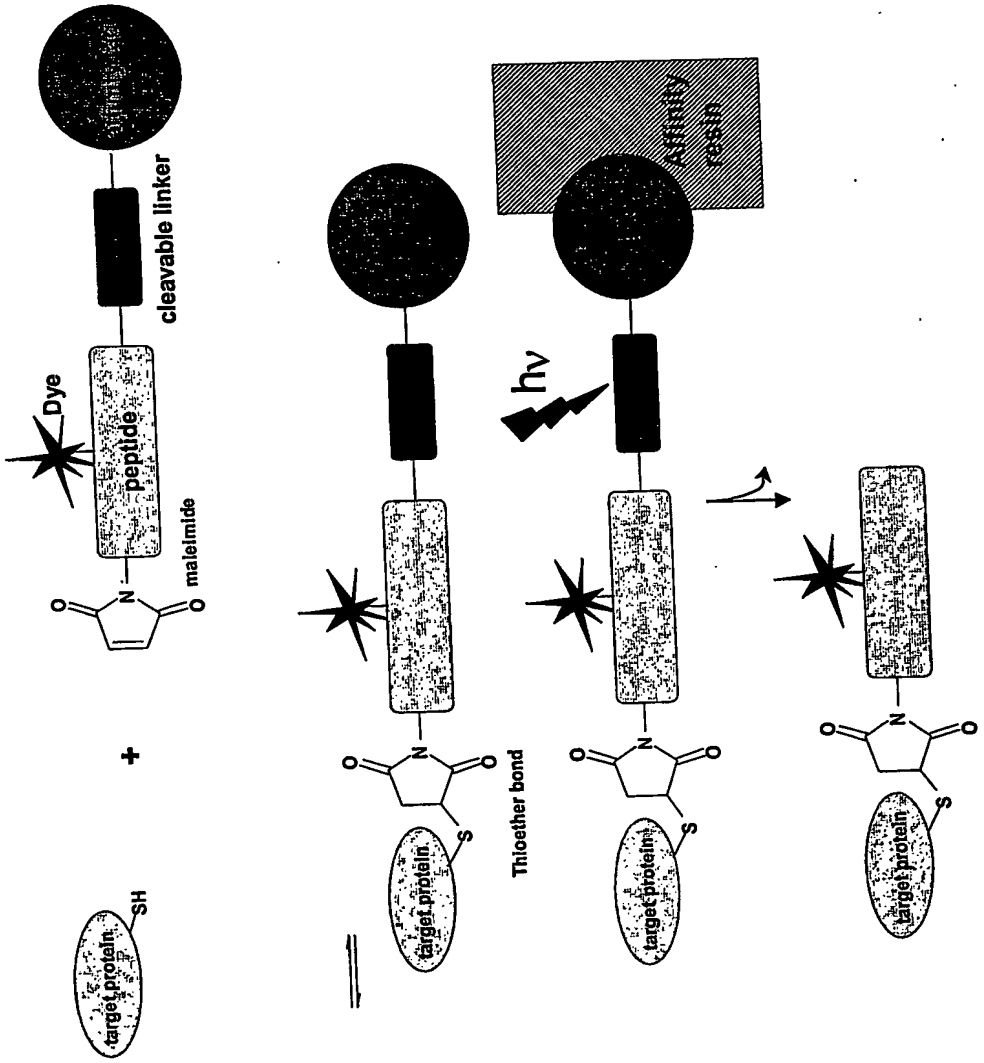

cleavable linker
Dye
peptide
maleimide
target protein
SH
Affinity resin
hν
Thioether bond
target protein
target protein
target protein

Figure 7


cleavable linker
Dye
peptide
maleimide
target protein
SH
+
Thioether bond
Affinity resin
hν
target protein
target protein
target protein

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